Pathogen Reduction of Fresh Whole Blood for Military and Civilian Use

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Hemorrhage resulting from combat-related injuries is the most common cause of potentially preventable deaths in military operations. Evidence indicates that the use of fresh whole blood (FWB) during massive transfusion (>10 units) is independently associated with improved survival[1]. The use of warm FWB currently persists only in emergency life-threatening scenarios when tested, stored blood components are not available, such as combat casualties. Risks of non-leukoreduced FWB transfusions include the transmission of infectious agents due to prior exposures or exposures in theater, alloimmunization to donor antigens and the potential for microchimerism. These risks may be mitigated in large part by a process capable of pathogen reduction and leukocyte inactivation. Such a technique, Pathogen Reduction Technology (PRT), can increase the safety of FWB and provide both an adequate and safe blood supply in a combat environment. This presentation will review work in progress on the development of such a system to treat fresh whole blood, which is capable of reducing infectious pathogen loads of bacteria, viruses, and parasites and inactivating white blood cells. This process is based on the use of riboflavin (vitamin B2) and UV light and relies on a photochemical process capable of disrupting nucleic acid replication processes in pathogens and white cells. Pre-clinical evaluations of device performance include tests of blood component quality, as well as tests of WBC inactivation, of virus reduction, and of bacteria reduction. The device is currently in clinical evaluation in the United States under an FDA approved Investigational Device Exemption. Early results with this system from pre-clinical and clinical studies will be provided. This work is supported through grants provided by the US Department of Defense.

1.0 INTRODUCTION

Whole blood was the first blood product to be transfused for therapeutic purposes. In military combat situations, it continues to be a valued therapeutic tool. Hemorrhage resulting from combat-related injuries is the most common cause of potentially preventable deaths in military operations. Evidence indicates that the use of fresh whole blood (FWB) during massive transfusion (>10 units) is independently associated with improved survival [1]. The use of warm FWB currently persists only in emergency life-threatening scenarios when tested, stored blood components are not available, such as combat casualties [2]. Risks of non-leukoreduced FWB transfusions include the transmission of infectious agents due to exposures to these agents either before or during deployment in the field, transfusion-associated graft-versus-host-disease (TA-GvHD), alloimmunization to donor antigens and the potential for microchimerism. These risks may be mitigated in large part by a process capable of pathogen reduction and leukocyte inactivation. Such a technique could increase the safety of FWB and thus provide both an adequate and safe blood supply in the combat environment. This presentation will review work in progress on the development of such a system to treat fresh whole blood, which is capable of reducing infectious pathogen loads of bacteria, viruses, and parasites

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14. ABSTRACT

Hemorrhage resulting from combat-related injuries is the most common cause of potentially preventable deaths in military operations. Evidence indicates that the use of fresh whole blood (FWB) during massive transfusion (>10 units) is independently associated with improved survival1. The use of warm FWB currently persists only in emergency life-threatening scenarios when tested, stored blood components are not available, such as combat casualties. Risks of non-leukoreduced FWB transfusions include the transmission of infectious agents due to prior exposures or exposures in theater, alloimmunization to donor antigens and the potential for microchimerism. These risks may be mitigated in large part by a process capable of pathogen reduction and leukocyte inactivation. Such a technique, Pathogen Reduction Technology (PRT), can increase the safety of FWB and provide both an adequate and safe blood supply in a combat environment. This presentation will review work in progress on the development of such a system to treat fresh whole blood, which is capable of reducing infectious pathogen loads of bacteria, viruses, and parasites and inactivating white blood cells. This process is based on the use of riboflavin (vitamin B2) and UV light and relies on a photochemical process capable of disrupting nucleic acid replication processes in pathogens and white cells. Pre-clinical evaluations of device performance include tests of blood component quality, as well as tests of WBC inactivation, of virus reduction, and of bacteria reduction. The device is currently in clinical evaluation in the United States under an FDA approved Investigational Device Exemption. Early results with this system from pre-clinical and clinical studies will be provided. This work is supported through grants provided by the US Department of Defense.

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and inactivating white blood cells (WBC). Treatment with the system is based on the use of riboflavin (vitamin B2) and UV light and relies on a photochemical process, which is capable of disrupting nucleic acid replication in pathogens and white cells.

2.0 DEVICE DESCRIPTION

The prototype Mirasol® System for Whole Blood is adapted from the CE-marked Mirasol PRT System for Platelets and Plasma. The device consists of an illuminator and a disposable kit, illustrated in Figure 1. For treatment with the system, a unit of whole blood (approximately 460 to 560 mL, with anticoagulant) is transferred to the illumination bag and mixed with the riboflavin solution (35 mL of 500 µM riboflavin in 0.9% NaCl). The mixture is then placed in the illuminator and exposed to UV light. The dose of UV light is normalized to account for the presence of the RBCs and sample volume (weight) and is reported in units of J/mL_{RBC}. Energy dose, mixing and product temperature are measured and recorded throughout the process on an on-board microprocessor/controller. Treatment times range from 40-60 minutes based on product size.

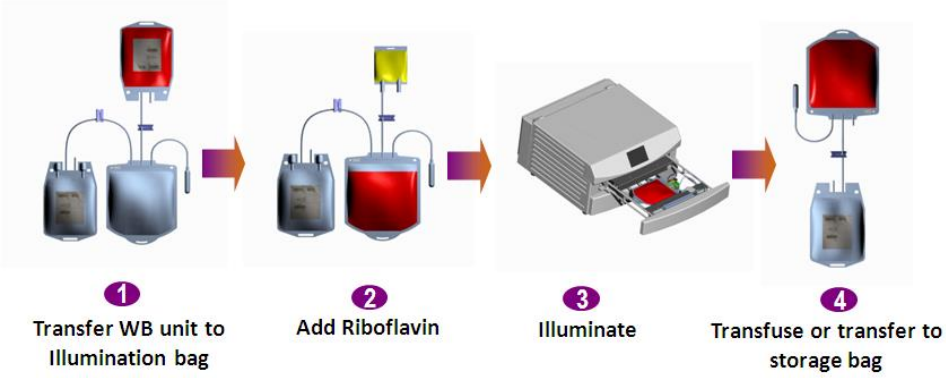

Figure 1: Treatment with the Mirasol System for Whole Blood.

3.0 PRE-CLINICAL RESULTS

Proof-of-concept testing for the Mirasol System for Whole Blood involved evaluations of WBC inactivation, virus reduction, bacteria reduction, component quality, and whole blood quality after treatment of the whole blood with varying UV-light energy doses. This information was utilized to select a light energy dose which afforded adequate levels of pathogen reduction and white cell inactivation, while also maintaining cellular and protein component functionality.

3.1 Virus Reduction

Several model viruses were used to assess the ability of this system to inactivate viruses in blood products. Efficacy was determined by measuring the residual levels of infectivity present in the samples after treatment and by comparing this to levels of infectivity in the untreated starting material. These values were used to calculate a reduction factor. Units of whole blood were spiked with enveloped (VSV, IBR) and non-enveloped (CPV, BTV, HAV) viruses as these are representative of the two major virus types. Virus inactivation as a function of energy delivered was evaluated in the range of 40-110 J/mL_{RBC}. Increasing virus inactivation with increasing energy was observed in the range of 40-110 J/mL_{RBC} (Figures 3 and 4).

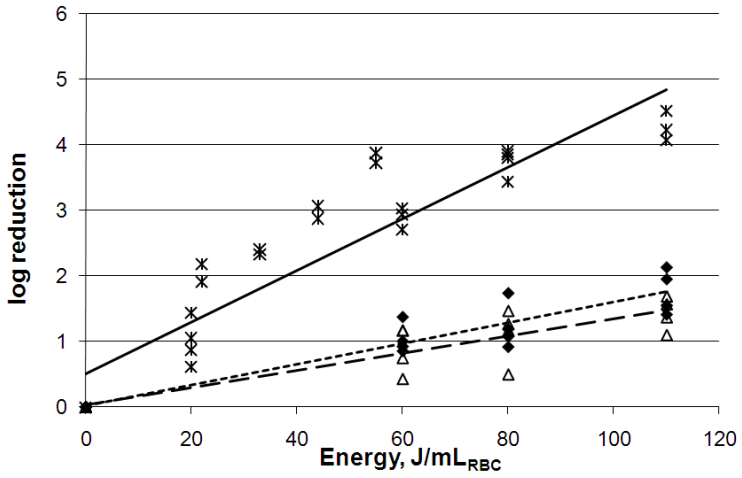

Figure 3: Reduction of non-enveloped virus as a function of energy. Symbols correspond to data, the lines to linear regressions of the data. Solid line with asterisks: CPV; dotted line with filled diamonds: HAV; dashed line with open triangles: BTV.

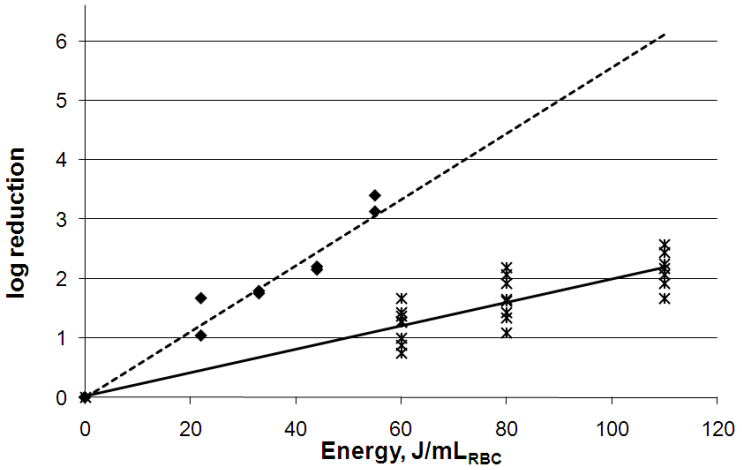

Figure 4: Reduction of enveloped virus as a function of energy. Symbols correspond to data, the lines to linear regressions of the data. Solid line with asterisks: IBR; dotted line with filled diamonds: VSV.

3.2 Bacteria Reduction

The ability of the system to reduce levels of infectious bacteria load was evaluated by measuring the ability of the system to sterilize products spiked with several strains of pathogenic bacteria. Sterility was assessed by culturing samples removed from treated products after storage at room temperature for up to 7 days. Bacterial growth curves, in cases of breakthrough, were also assessed to establish the extent of a bacteriostatic effect (Figure 5). Initially, evaluation of bacteria reduction was performed as a function of dose response over the range of 40 – 110 J/mL$_{RBC}$. Follow-up experiments focused on evaluating performance after treatment with 80 J/mL$_{RBC}$. Results are summarized in Table 1.

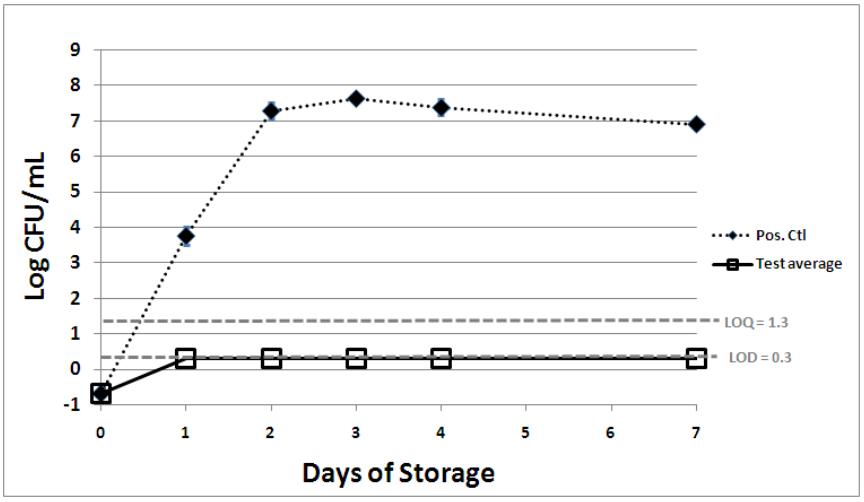

Figure 5: Growth rate of B. cereus in treated and positive control whole blood. LOD = limit of detection of the assay used to measure bacteria titer.

Table 1: Low-titer bacteria reduction after treatment with 80 J/mL$_{RBC}$ in the Mirasol System.

Strain of bacteria tested	# of units positive/ # of units tested
Serratia marcescens	0/3
Yersinia enterocolitica	0/15
Escherichia coli	0/2
Klebsiella pneumoniae	0/3
Acinetobacter baumannii	1/9
Serratia liquefaciens	1/10
Staphylococcus aureus	1/2
Bacillus cereus	2/8
Streptococcus pyogenes	4/10
Staphylococcus epidermidis	15/22

3.3 Parasite Reduction

Several experiments to test the effectiveness of the process for inactivating parasites are ongoing or are planned. These studies are of particular importance because tests for parasitic agents are usually not available due to the lack of suitable diagnostic tests coupled with the unique characteristics of these agents, which can evade detection in blood. Exposure of military donors in the field to these agents can be of particular concern [3,4]. Tests of the reduction of a transfusion-transmissible parasite, *Trypanosmoa cruzi*, with the Mirasol System for Whole Blood are ongoing. Tests of *Babesia microti*, *Leishmania donovani*, *Plasmodium falciparum*, and *Anaplasma phagocytophilum* reduction will occur over the next three years.

3.4 WBC Inactivation

WBC in blood products can induce multiple adverse effects in the transfusion recipient including life-threatening TA-GvHD, cytokine production and alloimmunization [5,6,7]. Leukoreduction and gamma

irradiation are used to lessen these effects but are not always available or practical for field implementation. Those measures also are only partially effective in alleviating adverse effects due to transfused WBC [8,9].

To assess inactivation, WBCs were isolated from treated whole blood products and WBC functionality was assessed. The phenotype of the cells does not change immediately after treatment. WBC activation by PMA, as measured by CD69 expression, was completely prevented at all energies tested (Figure 6). Proliferation in response to PHA and anti-CD3 plus anti-CD28 antibodies was completely blocked at the highest energy applied (Figure 7). In a mixed lymphocyte culture proliferation of treated WBCs in response to allogeneic stimulator cells (allorecognition) or proliferation of untreated cells in response to treated stimulator cells (allostimulation) was also inhibited, indicating that Mirasol treatment impairs antigen presentation in addition to cell division (Figure 8 and 9). Production of inflammatory and TH1/TH2 cytokines in response to LPS and anti-CD3 plus anti-CD28 antibodies was completely inhibited at the higher energies used (data not shown).

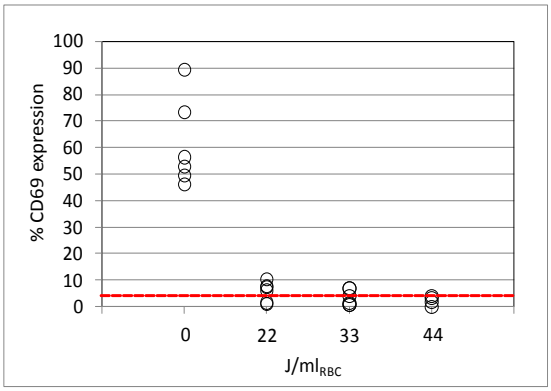

Figure 6: Inhibition of WBC activation in response to PMA, treated WBCs (22, 33 or 44 J/mL$_{RBC}$) and untreated control WBCs (0 J/mL$_{RBC}$). CD69 expression was measured by flow cytometry. The dashed line represents the limit of detection of the assay.

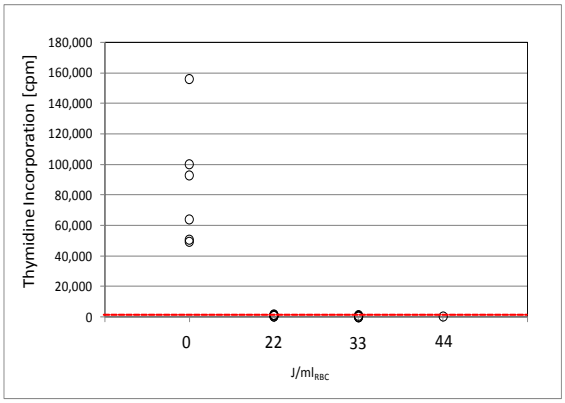

Figure 7: Inhibition of WBC proliferation in response to PHA, in treated WBCs (22, 33 or 44 J/mL$_{RBC}$) and untreated control WBCs (0 J/mL$_{RBC}$). Proliferation was measured by thymidine incorporation. The dashed line represents the limit of detection of the assay.

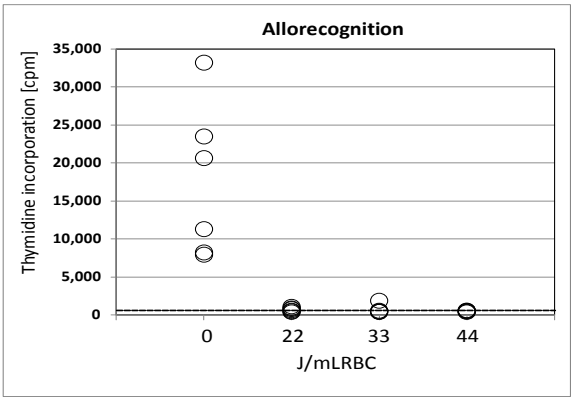

Figure 8: Results for allorecognition by treated WBCs (22, 33 or 44 J/mL$_{RBC}$) and untreated control WBCs (0 J/mL$_{RBC}$). Proliferation in a mixed lymphocyte culture was measured by thymidine incorporation. Dashed line represents the limit of detection of the assay.

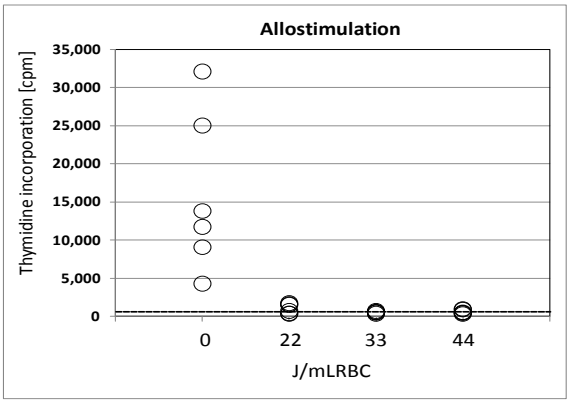

Figure 9: Results for allostimulation by treated WBCs (22, 33 or 44 J/mL$_{RBC}$) and untreated control WBCs (0 J/mL$_{RBC}$). Proliferation in a mixed lymphocyte culture was measured by thymidine incorporation. Dashed line represents the limit of detection of the assay.

The functionality of treated WBCs was also assessed in a mouse model that utilizes xenogeneic GvHD responses of human cells when injected into immunodeficient murine recipients ($Rag^{-/-}\gamma c^{-/-}$ double knockout mice). The results of this work showed prevention of GvHD in animals transfused with treated products, as evidenced by lack of GvHD development, human cell engraftment (Figure 10), antibody production, and increase in human cytokine levels (Figure 11).

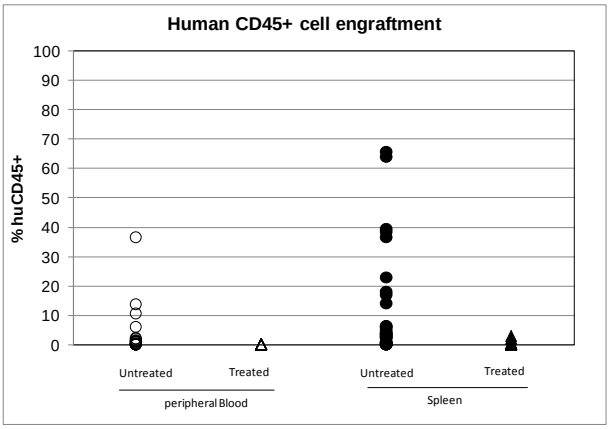

Figure 10: Human CD45+ cell engraftment in the mice used in the xGvHD study.

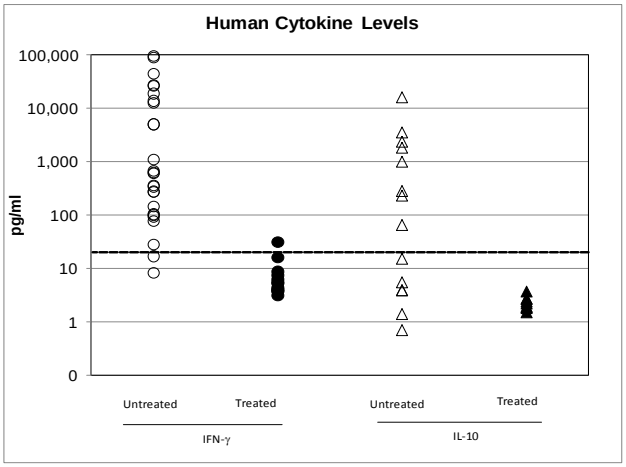

Figure 11: Human cytokine levels in the mice used in the xGvHD study. Dashed line represents the limit of quantification of the assay.

3.5 Whole Blood Quality and Function

In order to assess the quality and functionality of whole blood, units were collected in CPDA-1, and treated with varying energy doses in the Mirasol System and then stored at room temperature, without agitation, for at least 24 hours (range of 1 to 7 days). The results of the studies indicated that 80 J/mL_{RBC} would provide a balance of acceptable blood quality and pathogen reduction. Additional studies were conducted to verify the performance of the system with 80 J/mL_{RBC} light delivery. Samples were removed to assess hemolysis, osmotic fragility, CBC, clinical chemistry, plasma function (PT, APTT, the activity of representative factors), and platelet function (TEG and ImpactR).

Free hemoglobin remained below the limits of detection (30 mg/dL) in treated and control units throughout 7 days of room-temperature storage. Methemoglobin values were elevated post-treatment (1.7% + 0.8%), and were at background levels (0.7%) after one day of storage. **Table 2** displays values for mean osmotic fragility; on Day 0 only, treated units were more fragile than the unpaired controls. On all other storage days, no

significant differences in osmotic fragility were observed between treated and control units. **Table 3** displays value for sodium and potassium concentrations. The potassium concentration increases, and the sodium decreases, more rapidly in the treated whole blood than in the controls.

Table 2: Mean Osmotic Fragility for Treated and Control RBCs during Storage.

Storage Day	MOF for control units (n=4)	MOF for treated units (n=8)
Day 0	0.468 ± 0.008	0.490 ± 0.018[a]
Day 1	0.503 ± 0.011[b]	0.504 ± 0.024[b]
Day 2	0.510 ± 0.006[b]	0.520 ± 0.019[b]
Day 5	0.551 ± 0.015[b]	0.549 ± 0.023[b]
Day 7	0.569 ± 0.015[b]	0.542 ± 0.046[b]

[a]Significantly different from control values.
[b]Significantly different from corresponding Day 0

Table 3: Clinical Chemistry Measurements during Storage.

	Storage Day	Na+ (mmol /L)	K+ (mmol/L)
Control (n=4)	Day 0	154 ± 1.4	3.2 ± 0.1
	Day 1	156 ± 1.5	3.8 ± 0.1
	Day 2	157 ± 1.5	5.1 ± 0.2
	Day 5	155 ± 1.5	9.0 ± 0.5
	Day 7	153 ± 1.7	11.3 ± 0.7
Treated (n=8)	Day 0	156 ± 1.9	3.3 ± 0.4
	Day 1	156 ± 1.6	5.1 ± 0.6[a]
	Day 2	155 ± 1.7	7.5 ± 0.8[a]
	Day 5	151 ± 2.0[a]	14.3 ± 1.2[a]
	Day 7	147 ± 2.4[a]	18.4 ± 1.5[a]

[a]Significantly different from controls.

Post-treatment plasma from eight treated units of whole blood, and plasma from three untreated units, was assayed for Fibrinogen, Factor V, Factor VIII, Factor XI, and total protein. In a separate experiment, after illumination the whole blood was transferred to the Mirasol storage bag for room temperature storage. Samples from eight units of treated whole blood and 4 units of untreated whole blood were assayed post-illumination and after one and two day's storage for prothrombin time (PT) and activated partial thromboplastin time (aPTT).

Table 4 displays the data for Fibrinogen concentration, Factor V, VII, and XI activity, and total protein concentration; these values are corrected for dilution with riboflavin solution. Total protein concentrations were equivalent in test and control plasma. Post-illumination values for Fibrinogen, Factor V, Factor VIII and Factor XI were higher in the controls than in the treated plasma, but all values were within normal ranges. **Table 5** displays the data for prothrombin time (PT) and activated partial thromboplastin time (aPTT) on days

0, 1 and 2 of room temperature storage. PT and aPTT values were greater in treated units than in controls, post-illumination and after one day of storage. Values for aPTT are in the range of normal values, and for PT are slightly elevated (for treated units).

Table 4: Plasma Quality: Fibrinogen, Factor V, Factor VIII, Factor XI.

		Fibrinogen (mg/dL)	Factor V (%)	Factor VIII (%)	Factor XI (%)	Total Protein (g/dL)
Control (n=3)	Day 0	255 ± 29	86 ± 9	79 ± 7	126 ± 8	5.5 ± 0.7
80 J/mL$_{RBC}$ (n=8)	Pre-illum	292 ± 25	89 ± 16	98 ± 18	108 ± 19	5.5 ± 0.3
	Day 0	214 ± 15	68 ± 13	70 ± 12[a]	85 ± 15	5.7 ± 0.3

[a]Significant difference ($p < 0.05$) between treated and control, t-test for unpaired samples, unequal variance

Table 5: Plasma Function: PT and APTT.

	Control (n=4)				80 J/mL$_{RBC}$ (n=8)			
	Pre-illum	**Day 0**	**Day 1**	**Day 2**	**Pre-illum**	**Day 0**	**Day 1**	**Day 2**
PT (sec)	N/A	13.4 ± 0.6	13.6 ± 0.6	13.9 ± 0.5	13.9 ± 0.6[a]	15.6 ± 0.7[a]	16.0 ± 0.9[a]	16.4 ± 0.8[a]
APTT (sec)	N/A	26.4 ± 2.2	27.0 ± 1.6	28.2 ± 2.3	25.2 ± 0.9[a]	31.3 ± 1.3[a]	32.7 ± 1.4[a]	33.1 ± 1.3[a]

[a]Significant difference ($p < 0.05$) between treated and control, t-test for unpaired samples, unequal variance

Platelet function was evaluated in test and control units of whole blood. The platelet function assays used were the ImpactR and thromboelastography (TEG). The Impact-R tests platelet adhesion and aggregation in anti-coagulated whole blood under arterial flow conditions. The results are expressed as the percentage of the well surface covered by platelet aggregates (% SC) which represents platelet adhesion, and the average size of the aggregates (AS in μm^2) which represents platelet aggregation.

TEG analysis evaluates clot formation by rotating a cup, containing the blood sample, to imitate venous flow and activate coagulation. The speed and strength of clot formation are represented with four parameters: R, k, MA and alpha. R (reaction time) is the elapsed time until clot formation in the analyzer – shorter R times are preferred. The alpha angle represents the rate of clot growth – smaller α values are preferred. The k-value is a measure of the speed required to reach a certain level of clot strength, and is closely related to alpha and to MA. MA (Maximal amplitude) is a measure of the strength of the clot formed, and is a measurement of viscosity – larger MA values are preferred.

For these unpaired treated and control articles, no statistically significant differences were observed for the Impact R measurements throughout storage (Table 6). For the Day 1 and Day 7 TEG measurements (Table 7),

no statistically significant differences were observed in values for MA, K and alpha. R values on Day 7 were significantly greater ($p < 0.05$) for treated units, but were not different on Day 1. Overall, the platelet function measurements indicate that platelets in treated whole blood display function that is equivalent to untreated platelets after 24 hours of room temperature storage. The PT and aPTT measurements in Table 9 were obtained from the same units as those evaluated with TEG. The differences in PT and aPTT did not impact clot formation, as evaluated by TEG (Table 11).

Table 6: Platelet Function Measurements – ImpactR.

		Day 0	Day 1	Day 2	Day 5	Day 7
Surface Coverage, %	**Control (n=4)**	8.4 ± 2.2	7.1 ± 1.9	7.2 ± 1.4	3.4 ± 2.3	3.4 ± 2.3
	Treated (n=8)	9.1 ± 1.8	7.2 ± 1.7	7.8 ± 2.1	3.8 ± 2.3	2.6 ± 2.4
Average Aggregate size, μm^2	**Control (n=4)**	29.3 ± 4.3	24.1 ± 1.1	25.6 ± 1.7	26.1 ± 2.7	25.9 ± 2.4
	Treated (n=8)	34.8 ± 9.6	23.5 ± 1.6	25.0 ± 2.3	23.6 ± 4.0	24.3 ± 2.3

Table 7: Platelet Function Measurements – TEG.

		Day 1	Day 7
R, min	**Control (n=4)**	7.8 ± 2.0	13.3 ± 1.7
	Treated (n=8)	6.6 ± 0.9	16.0 ± 2.0[a]
K, min	**Control (n=4)**	1.9 ± 0.2	2.2 ± 0.6
	Treated (n=8)	1.9 ± 0.3	2.9 ± 0.6
Alpha, degrees	**Control (n=4)**	63.0 ± 3.1	57.7 ± 7.1
	Treated (n=8)	63.3 ± 3.5	51.2 ± 4.9
MA, mm	**Control (n=4)**	63.0 ± 5.2	37.4 ± 8.7
	Treated (n=8)	62.1 ± 2.2	43.2 ± 6.3

[a]Significant difference ($p< 0.05$) between treated and control, t-test for unpaired samples, unequal variance

4.0 CLINICAL STUDIES: RESULTS FROM FIRST RECOVERY AND SURVIVAL STUDY

The Mirasol System for Whole Blood has been tested in a feasibility clinical study (the IMPROVE study) with healthy volunteers, under an FDA-approved Investigational Device Exemption (IDE) and with funds from the U.S. Department of Defense. The overall objective of the IMPROVE Study was to establish the correlation between in vitro measures of cell quality and in vivo recovery and survival of radiolabelled cells in circulation. These correlations were derived by comparing three different illumination energies and

subsequent extended storage for 42 days. In the study, each healthy volunteer donated one unit of whole blood. The unit of whole blood was not leukoreduced, and was treated with the Mirasol System and then separated into components (pRBCs in AS-3, platelet concentrates (PC), and fresh frozen plasma (FFP)). Each component was stored at the appropriate storage condition: 4°C for pRBCs, 22°C for PC, and -20°C for FFP. All components were sampled on the day of preparation, and again at the end of storage (Day 42 for RBCs, Day 5 for PCs, and Day 28 for FFP).

The *in vitro* testing performed on the units included assessments of WBC inactivation and evaluations of pRBC, PC, and FFP quality. For the *in vivo* portion of the study, each volunteer received an aliquot (~ 12 mL) of 51-chromium labelled RBCs that had been stored for 42 days after treatment with the Mirasol System. Blood samples from the volunteers were withdrawn during the first day post-infusion to determine the 24-hour recovery values, and for 4 weeks after infusion to determine survival of the labelled cells in circulation.

Spearman Correlations of > 0.7 were identified between 24-hour recovery values and two of the Day 42 *in vitro* variables: % hemolysis and ATP (Figure 12). The highest correlations (R_s > 0.7) with T50 Survival results were found with ATP and pCO2 (Day 42 values) (Figure 13).These observations were consistent with those reported previously for untreated red cells.[10]

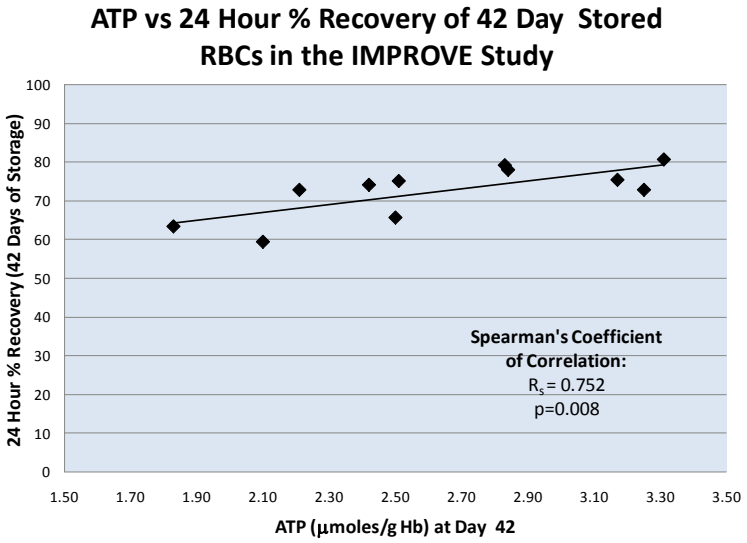

Figure 12: Percent recovery of 51-chromium labelled RBCs as a function of ATP.

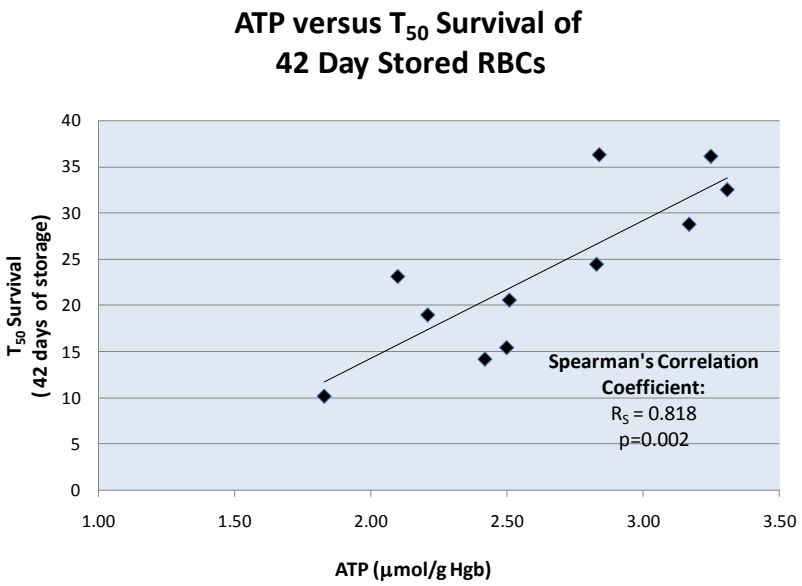

Figure 13: Survival Half-Life of 51-chromium labelled RBCs as a function of ATP content.

Using the correlations observed in this study, combined with in vitro measures of cell quality following treatment with a UV light dose of 80J/mL$_{RBC}$ and storage at RT, it is possible to predict the performance of the treated red cells and evaluate this relative to FDA standards for red cell performance (Figure 14). This standard requires a recovery in circulation of 75% of the transfused cells at 24 Hours post-infusion. The accuracy of this predicted outcome will be verified through additional clinical evaluation of the product in both healthy subjects and patients.

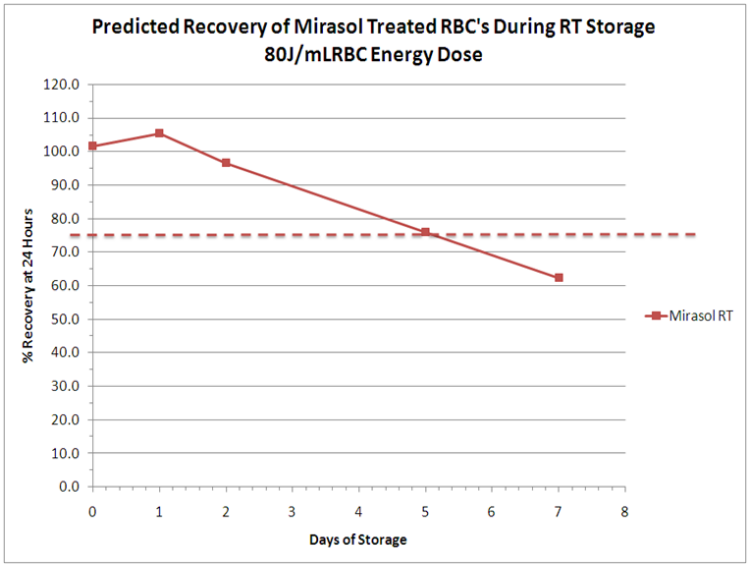

Figure 14: Predicted recovery of Mirasol-treated RBCs during room temperature storage after treatment with the Mirasol System for Whole Blood and 80 J/mL$_{RBC}$ UV light energy.

5.0 SUMMARY/CONCLUSION

A method to treat whole blood to reduce the infectious load of viruses, bacteria and parasites is in development. This technology can have tremendous utility in the military field hospital setting, providing a means to greatly improve the safety and supply of blood for combat casualty care. This device, known as the Mirasol System for Whole Blood, is intended for use in the field setting where conventional methods of blood banking are not practical or feasible but where the need for safe blood products to support casualty care is significant. This approach may supplement existing test methods and act as an added barrier against disease transmission in the combat support setting, where testing may be unavailable, impractical or not feasible. Similarly, the ability of this process to inactivate donor WBC has the potential benefit of reducing or eliminating several adverse events mediated by donor WBC in transfused blood. This technology may thus serve as a means to practically and efficiently deliver blood products equivalent to or even exceeding the safety available from tested, leukoreduced and gamma irradiated components commonly employed in the civilian setting, thus delivering a premium of care in the field to injured combatants.

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